Acyclic retinoid NIK-333 accelerates liver regeneration and lowers serum transaminase activities in 70% partially hepatectomized rats, in vivo

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Abstract: The effect of an acyclic synthetic retinoid analogue NIK-333, on the restoration of liver mass and recovery of liver function after 70% partial hepatectomy, was compared with natural retinoids in rats in vivo. NIK-333 (0.4 mg/kg/day, p.o.)- and all-*trans*-retinoic acid (ATRA: 4 mg/kg/day, p.o.)-treated rats showed an approx. 1.3- and 1.2-fold increase in liver-to-body weight ratio, respectively, compared to solvent-administered control rats on day 3 after 70% partial hepatectomy. Accordingly, 5-bromo-2'-deoxyuridine (BrdU)-labeling index in the regenerating liver was significantly higher in NIK-333- and ATRA-treated rats compared with control rats on days 0.5 and 1. However, retinol (40 mg/kg/day, p.o.) did not significantly increase either the liver-to-body weight ratio or the BrdU labeling index. In control rats, liver-related serum transaminase activities such as alanine aminotransferase and aspartate aminotransferase, were rapidly elevated on day 1 and then decreased to near pre-operative levels on day 5 following 70% partial hepatectomy. NIK-333 significantly lowered serum transaminases on days 1 and 3 after 70% partial hepatectomy compared with solvent-administered control rats. The transaminase-lowering effect of NIK-333 was more effective than that of ATRA. Retinol did not significantly decrease serum transaminases compared with the control. These results demonstrate that of the three retinoids, NIK-333 was the most potent in promoting the regeneration of

liver mass and function with full recovery after 70% partial hepatectomy.

Key words: Partial hepatectomy; Liver regeneration; Serum transaminase; Acyclic retinoid (NIK-333); All-*trans* retinoic acid; Retinol

1. Introduction

Vitamin A and its natural and synthetic derivatives are known as retinoids (Murphy et al., 2007). Vitamin A alcohol is the physiological form of vitamin A. Retinoic acid is a natural product derived from the metabolism of retinol. Retinoic acid is also known as tretinoin or all-*trans*-retinoic acid (ATRA). ATRA is the main signaling retinoid in the body and is vital for biological functions such as embryogenesis, growth, and differentiation, as well as for vision and reproduction (Dragnev et al., 2000). In addition, retinoids have been shown to exert an anticarcinogenic effect through the suppression of cell cycle, and the induction of apoptosis and/or differentiation (de Almeida Vasconcelos Fonseca et al., 2005; Kagawa et al., 2004; Nakamura et al., 1996; Suzuki et al., 2002).

Acyclic retinoid (NIK-333), a novel synthetic vitamin A analogue, has a slightly different chemical structure from natural retinoic acid (Araki et al., 1995; Nakamura et al., 1995). Acyclic retinoids are considered to act in a similar way to natural retinoic acid, because they bind to cellular retinoic acid-binding protein with strong binding affinity (Araki et al., 1995; Chambon, 1996; Garttini et al., 2007). A number of retinoids have been assessed with the aim of determining their role in chemoprophylaxis of chemical carcinogens. It has been reported that NIK-333 retains chemopreventive activity and is less toxic than ATRA (Muto et al., 1996).

Both natural and synthetic retinoids have been used for the treatment of patients with breast carcinomas, head and neck squamous cell carcinomas, and acute leukemia (Hansen et al., 2000).

However, in spite of the proposed therapeutic effects of ATRA and NIK-333, very little data is available on its effects on normal (non-tumorous) liver cell proliferation. In contrast to malignant cell lines, it has been reported that normal parenchymal hepatocytes significantly proliferate during ATRA treatment. For instance, Ledda-Columbano et al. showed that ATRA administration in the diet increased DNA synthesis in mouse liver, at 1 and 2 weeks. Furthermore, increase in mitotic index paralleled that of bromodeoxyuridine incorporation (Ledda-Columbano et al., 2004; Ohtake et al., 2006). In rat liver, however, retinoic acid has been shown to inhibit regeneration after partial hepatectomy, most probably through repression of the expression of c-*fos* and c-*jun* (Ozeki and Tsukamoto, 1999). ATRA is reported to be a potent inhibitor of DNA synthesis in the primary cultures of hepatocytes from mature rats (Ikeda and Fujiwara, 1993). Hence, the regulation of hepatocyte proliferation by natural and synthetic retinoids is far more complex than initially thought and is often controversial. It remains to be clarified the effects that retinoids have on liver regeneration and the mechanisms involved.

The purpose of this study was to evaluate the effects of NIK-333 on the

restoration of liver mass and recovery of liver function, compared with the effects of natural retinoids (ATRA and retinol), in 70% partially hepatectomized rats in vivo. In order to examine whether retinoids would protect the integrity of the liver after 70% partial hepatectomy, we measured the activities of liver-related cytosolic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in sera.

2. Materials and Methods

2.1. Animals

Seven-week-old male Wistar rats weighing 200 g were obtained from Tokyo Experimental Animal Co. (Tokyo, Japan). The rats were housed in a controlled temperature of 24 °C under a 12 h light-dark cycle, and were fed standard laboratory chow and water ad libitum. The rats were handled humanely in accordance with the Guidelines for the Care and Use of Laboratory Animals of Josai University.

2.2. 70% partial hepatectomy and drug administration

70% (two-thirds) partial hepatectomy was performed under ether anesthesia according to the method of Higgins and Anderson (Higgins and Anderson, 1931), with minor modifications. In brief, the left lateral and median hepatic lobes, constituting approximately 70% of the total liver weight, were ligated and resected. In sham-operated controls that were similarly anesthetized, the livers were briefly removed from the peritoneal cavity, but not tied or excised.

The rats were randomly divided into 4 groups that received either a solvent (soybean oil), NIK-333, all-*trans* retinoic acid, or retinol. NIK-333 and natural retinoids were emulsified in soybean oil. Each group was treated with oral administration of either a solvent (control: soybean oil, 4

mL/kg), NIK-333 (0.4 mg/kg), all-*trans* retinoic acid (4.0 mg/kg), or retinol (40 mg/kg), once per day (at 10 A.M.). The administration was repeated and continued for 1 to 14 days unless otherwise indicated.

2.3. Determination of liver regeneration

All rats were killed at indicated time points after 70% partial hepatectomy under ether anesthesia. The regenerating liver was excised, and moist liver weight after regeneration and body weight was measured. The ratio of total liver weight after regeneration was normalized to body weight and used as an indicator of liver regeneration (Assy and Minuk, 1997).

2.4. 5-Bromo-2'-deoxyuridine (BrdU) incorporation

Liver regeneration monitored by mitotic index demonstrates nuclear 5-bromo-2'-deoxyuridine (BrdU) incorporation into hepatocyte DNA after 70% partial hepatectomy, according to a method previously described (Gratzner, 1982; Ishiki et al., 1992). BrdU, a thymidine analogue, is incorporated into hepatocyte nuclei during DNA synthesis (S phase in cell cycle). BrdU incorporation is a useful indicator of the effect of growth factors on cell proliferation in a variety of experimental systems. BrdU (50 mg/kg, i.p.) was injected into rats and 2 h later the rats were anesthetized with

ether and the livers removed. In brief, livers were fixed in 10% neutralized formalin and embedded in paraffin. Paraffin-embedded tissue sections (5 μm thick) were deparaffinized and stained with Hematoxylin. For assaying the labeling index, deparaffinized liver sections were incubated in 2N HCl for 30 min, washed several times with phosphate-buffered saline (pH7.4), and stained with anti-BrdU monoclonal antibody. BrdU was detected using avidin DH-biotinylated horseradish peroxidase complex according to the manufacturer's instructions (Exalpha, Biologicals, Inc., Maynard, USA). To determine the labeling index, the number of labeled nuclei out of 1000 nuclei, in a randomly selected field were counted under a light microscope.

To detect any histological changes on day 3 after 70% partial hepatectomy, sections of the regenerating liver were stained with Hematoxylin and Eosin.

2.5. Determination of serum transaminase activity

To establish whether retinoids could affect the integrity of the livers of 70% partially hepatectomized rats, we measured the activities of liver-related cytosolic enzymes in sera. After the remnant livers were removed, blood samples were quickly collected from the inferior vena cava. Enzyme assays were performed in normal rats with either NIK-333 (0.4 mg/kg), all-*trans* retinoic acid (4.0 mg/kg), retinol (40 mg/kg), or solvent (0.4 mL/kg) administration. Serum alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) activities were assayed using a commercially available diagnostic kit according to the manufacturer's instructions (Wako Pure Chemical Industries, LTD., Osaka, Japan). The enzyme reaction was achieved by adding the substrate with diaminobenzidine. One unit of enzyme activity was measured as the change in optical density of 0.001/min/ml of serum.

2.6. Materials

The acyclic retinoid, NIK-333 [(2*E*,4*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid] was provided by Tokyo New Drug Research Laboratories, Pharmaceutical Division, Kowa Co. (Higashimurayama, Tokyo, Japan). NIK-333 was emulsified in soybean oil. The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): all-*trans* retinoic acid (ATRA), retinol, and 5-bromo-2'-deoxyuridine (BrdU). BrdU was dissolved in 50% dimethylsulfoxide at 120 mg/ml (stock solution). Monoclonal anti-BrdU antibody was purchased from Exalpha Biologicals, Inc. (BrdU Immunohistochemistry Assay Kit, Maynard MA, USA), and monoclonal anti-transforming growth factor-β1 antibody was obtained from R & D Systems, Inc. (Minneapolis, MN, USA). The assay kits for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were

obtained from Wako Pure Chemical Industries, LTD, Osaka, Japan. All other reagents were of analytical grade.

2.7. Statistical analysis

Results are presented as the mean ± S.E.M. Group comparisons were performed by ANOVA for unpaired data followed by post hoc analysis using Dunnett's multiple comparison test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Time course-associated effects of NIK-333 and natural retinoids on liver regeneration

To examine the effect of NIK-333 on the regeneration of remnant liver after 70% partial hepatectomy, NIK-333 (0.4 mg/kg/day) was administered orally into rats and compared with the effects of all-*trans*-retinoic acid (ATRA : 4.0 mg/kg/day) and retinol (40 mg/kg/day). The ratio of total liver weight after regeneration normalized to body weight was used as an indicator of liver regeneration (Assay and Mink, 1997). After 70% partial hepatectomy, the liver-to-body weight ratio increased rapidly and reached an initial peak on day 5 in solvent-administered control rats, whereas the ratio increased by day 3 in NIK-333-treated rats (Fig. 1). The NIK-333-treated rats showed an approx. 1.3-fold increase over the solvent-administered controls on day 3 after 70% partial hepatectomy. This was statistically significant. The liver-to-body weight ratio peaked for a second time on day 10 after 70% partial hepatectomy and the moist liver weight was almost restored to pre-operative levels. Body weight did not differ significantly between the NIK-333-administered group and the solvent-administered controls from days 1 to 14. ATRA-treated rats (4.0 mg/kg, p.o.) reached a peak on day 7, and the time course that was associated with the effects of ATRA was similar to that of NIK-333, as

assessed by the liver-to-body weight ratio (Fig. 1). There were no significant differences in ratios calculated from the weight of remnant livers to body weight in rats given retinol (40 mg/kg, p.o.) once per day after 70% partial hepatectomy from days 1 to 14 when compared with solvent-administered controls (Fig.1).

3.2. Dose-dependent effects of NIK-333 and natural retinoids on liver regeneration

As the recovery of liver mass following 70% partial hepatectomy peaked 3 days after NIK-333-treatment, we decided to examine the dose-response effect of NIK-333 on the liver-to-body weight ratio at 3 days after the hepatectomy, and to compare it with that of all-*trans*-retinoic acid (ATRA) and retinol. As shown in Fig. 2, a bell-shaped dose-response curve indicates that a small dose of NIK-333 (0.4 mg/kg) stimulates liver regeneration, and that higher doses (4.0 and 40 mg/kg) are less effective. The maximal effect induced by NIK-333 was achieved with 0.4 mg/kg. Co-administration of monoclonal antibody to transforming growth factor-β1 (10 μg/kg/day, i.p.) with NIK-333 restored the typical dose-response relationship. A smaller dose of monoclonal antibody to transforming growth factor-β1 (5 μg/kg/day, i.p.) also restored the suppression induced by higher doses of NIK-333 (approx. 40%; data not shown). On the other hand, ATRA showed a typical

dose-response curve with a maximal dose of 4.0 mg/kg. Therefore, we used maximal effective doses of NIK-333 (0.4 mg/kg) and ATRA (4.0 mg/kg) in the following experiment. Retinol (0.01~40 mg/kg) did not significantly increase the liver-to-body weight ratio at any of the doses tested.

3.3. Light micrographs of typical liver sections from normal rats treated with or without NIK-333 or ATRA on day 3 after 70% partial hepatectomy.

As shown in Fig. 1, stimulation of liver regeneration by NIK-333 and ATRA was most evident on day 3 after 70% partial hepatectomy, according to our histological studies. Sections of regenerative livers were stained with Hematoxylin and Eosin to aid with the observation of histological changes on day 3 after 70% partial hepatectomy (Fig. 3). Histology shows that there are almost no morphological abnormalities such as cell-swelling, atypical changes of tissue, or infiltration of cells in sections of remnant regenerating livers treated with soybean oil (B), NIK-333 (C), or ATRA (D), compared with sham operated controls (A). We also examined whether histological changes would be present already on day 1 after 70% partial hepatectomy. We found no histological abnormalities in the sections of retinoid-treated livers compared with the sham-operated controls (data not shown).

3.4 Distribution of hepatocytes undergoing DNA synthesis on day 1 after 70% partial hepatectomy.

In Fig. 4, light micrographs reveal the typical distribution of hepatocytes undergoing DNA synthesis in the remnant liver of rats after sham operation (A), solvent (B), NIK-333 (C), or ATRA (D) treatment on day 1 after 70% partial hepatectomy. Hepatocytes were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 2 h in vivo and visualized by immunochemical staining, whereby dark spots indicate the hepatocytes that are undergoing DNA synthesis. Results show that NIK-333 and ATRA, but not sham-operated mice, significantly stimulate hepatocyte DNA synthesis. The BrdU labeling index of NIK-333-treated rats is significantly greater than that of ATRA-treated rats on day 1 after 70% partial hepatectomy. Hepatocytes undergoing DNA synthesis in both NIK-333- and ATRA-treated animals are randomly distributed within hepatic lobes.

3.5. Time course-associated effects of NIK-333 and ATRA on 5-bromo-2'-deoxyuridine (BrdU) labeling indexes on days 0 to 5 after 70% partial hepatectomy

The time course-associated effects of NIK-333 and ATRA on the BrdU labeling index in hepatic parenchymal cells is shown in Fig. 5. Even though the BrdU labeling index of sham-operated rat liver sections before 70%

partial hepatectomy was less than 0.1%, it moderately increased from the first day after the procedure in solvent-treated controls. Thereafter, it decreased to pre-operative levels at day 4 after 70% partial hepatectomy. In contrast, rats administered with 0.4 mg/kg/day NIK-333 showed a significant increase in BrdU labeling from 1 to 15% on day 1, which then declined to 5% on day 2. In both groups, the BrdU-labeling indexes returned to pre-operative levels on days 3, 4, and 5 after subjection to 70% partial hepatectomy. These results indicate that NIK-333 and ATRA significantly extended the mitogenic activity of hepatocytes in livers after 70% partial hepatectomy when compared with the control group. The effects from retinol were not statistically significant compared with solvent-treated controls (data not shown).

3.6. Time course-associated effects of NIK-333 and natural retinoids on serum transaminase activities

Of the known liver-related enzymes, transaminases (ALT and AST) in serum are good markers of liver injury or damage. To examine whether or not NIK-333, as well as natural retinoids, could affect liver integrity after partial hepatectomy, we measured ALT and AST activities in sera. As shown in Fig. 6, serum levels of ALT rapidly increased and peaked on day 1 in solvent-administered control rats after subjection to 70% partial

hepatectomy. ALT activity rapidly returned to pre-hepatectomy levels in solvent-treated control animals on day 3, and this continued for a further 11 days. However, NIK-333 (0.4 mg/kg/day, p.o.) inhibited the elevation of serum ALT activity induced by 70% partial hepatectomy already on day 1. The transaminase-lowering effect of NIK-333 was significant compared with solvent-treated control groups on days 1 and 3 after 70% partial hepatectomy. On the other hand, ATRA (4.0 mg/kg/day, p.o.) and retinol (40 mg/kg/day, p.o.) did not reduce serum ALT activity compared with the solvent-treated controls (Fig. 6).

Transaminase AST also increased significantly on day 1, after 70% partial hepatectomy in both control- and NIK-333-treated rats (Fig. 6). Administration of 0.4 mg//kg NIK-333, but not 4 mg/kg ATRA or 40 mg/kg retinol, also suppressed the elevated AST levels compared with the controls on day 1 after 70% partial hepatectomy.

3.7. Dose-dependent effect of NIK-333 and natural retinoids on serum transaminase activities

We next examined dose-response effects of NIK-333 and natural retinoids on liver-specific transaminase ALT and AST activities in serum on day 1 following 70% partial hepatectomy. As shown in Fig. 7, NIK-333 dose-dependently lowered both ALT and AST activities with a maximal

effect at 0.4 mg/kg/day. ATRA and retinol did not significantly affect either serum ALT or AST activities in 70% partially hepatectomized rats compared to solvent-treated controls.

4. Discussion

We evaluated whether or not exogenous administration of a novel synthetic analog of retinoid, NIK-333, or natural retinoids could stimulate liver regeneration in 70% partially hepatectomized rats in vivo. We found that restoration was almost complete by the 12th day in solvent-administered control rats, based on their liver-to-body weight ratio. In this study, administration of NIK-333 (0.4 mg/kg, p.o.) or ATRA (4.0 mg/kg, p.o.) significantly accelerated remnant liver regeneration, whereas this was not achieved with a higher dose of retinol (40 mg/kg, p.o.) compared to solvent-administered controls at selected time points (Fig. 1). Furthermore, ATRA dose-dependently stimulated liver regeneration (Fig. 2). In contrast, although low dose NIK-333 stimulated liver regeneration, higher doses of NIK-333 actually reduced liver regeneration on day 3 after 70% partial hepatectomy compared to its maximal effect. Yet, co-administration of NIK-333 at higher doses with monoclonal anti-TGF-β1 antibody significantly accelerated liver regeneration. Since TGF-β1 is

known to be a potent growth inhibitory factor in the liver (Kimura and Ogihara, 1999; Michalopoulos and DeFrances, 1997; Nishikawa et al., 1998), these results suggest that higher doses of NIK-333 stimulates endogenous secretion of TGF-β1 from non-parenchymal hepatocytes, thus inhibiting liver regeneration. In Fig. 2 we also show that among the retinoids tested here, NIK-333 is the most potent agent in enhancing liver regeneration in 70% partially hepatectomized rats.

Liver regeneration was also monitored by mitotic index, which demonstrates nuclear 2-bromo-5'-deoxyuridine (BrdU) incorporation into hepatocyte DNA after 70% partial hepatectomy, according to a previously established method (Gratzner, 1982; Ishiki et al., 1992). When looking at BrdU, administration of NIK-333 (0.4 mg/kg, p.o.) or ATRA (4.0 mg/kg, p.o.) significantly stimulated the BrdU labeling index at days 0.5 and 1 post-hepatectomy compared to solvent-treated controls, whereas treatment with retinol (40 mg/kg, p.o.) only slightly stimulated the BrdU labeling index (Fig. 5). To our knowledge, this is the first report showing that NIK-333 or ATRA are able to stimulate hepatocyte DNA synthesis during liver regeneration in 70% partially hepatectomized rats in vivo. The stimulatory effect of NIK-333 was more potent than that of ATRA. These results are consistent with our in vitro studies where NIK-333 is more potent than ATRA in stimulating hepatocyte DNA synthesis and

proliferation in primary hepatocyte cultures from adult rats (manuscript in preparation).

Acyclic retinoid binds to cytosolic retinoic acid binding protein (CRABP) and to the retinoic acid receptor (RAR), thereby transactivating genes through the retinoic acid responsive element or by binding to the retinoid X receptor (RXR) and transactivating genes via the retinoid X responsive element (Araki et al., 1995). The potent mitogenic activity of NIK-333 or ATRA in parenchymal hepatocytes seems to be one of the predominant mechanisms that act through the retinol receptor RAR and/or RXR (Chambon, 1996; Kastner et al., 1995). In support of this notion, Yang et al. showed that liver regeneration after partial hepatectomy is delayed in RXR-α receptor knockout mice (Yang et al., 2010). However, although the acyclic retinoid NIK-333 shares some characteristics with natural retinoids in vitro and in vivo, it has been reported that NIK-333 differs in its several biological effects from natural retinoids such as the all-*trans* and 9-cis retinoic acids (Komi et al., 2010; Sakabe et al., 2007; Shidoji and Ogawa, 2004; Tsurumi et al., 1993). In fact, administration of NIK-333 resulted in a biphasic dose-response relationship, whereas ATRA showed a typical dose-response relationship (Fig. 2). This prompted the authors to speculate that NIK-333 might mediate different mechanisms of action from the natural retinoids (e.g., ATRA and 9-cis retinoic acids) in stimulating normal

hepatocyte proliferation. This hypothesis remains to be elucidated.

From a therapeutic point of view, liver regeneration therapy for fulminant hepatitis is unsatisfactory and so sufficient liver regeneration cannot be expected after resection. Problems with hepatic failure arise seeing as liver function is markedly impaired in hepatectomized patients, from hepatitis, and from hepatic cancer. Hence, it is important to stimulate both the regeneration of mass and the recovery of function in the remnant liver after liver damage. However, at present, safe and efficacious therapies that suppress liver oncogenesis and promote proliferation of hepatocytes have yet to be discovered. Therefore, an agent should be developed that specifically promotes liver regeneration and recovery of liver function, and which is safe to use in the treatment of fulminant liver hepatitis, hepatic failure, and hepatic cancer resection.

Muto et al. demonstrated that one-year treatment with the acyclic retinoid NIK-333 significantly inhibited the recurrence of hepatocellular carcinoma by suppressing cell cycle progression and/or inducing apoptosis (Muto et al., 1996). Also, ATRA shows antitumor activity as strong as NIK-333 but with the absence of apoptosis (Okuno et al., 2004). It is known, however, that ATRA administration induces serious adverse effects such as retinoic acid syndrome (i.e., pyrexia, lung infiltration, intestinal pneumonia, renal failure, etc.). On the other hand, NIK-333 is a drug with a high safety

profile that does not cause impaired liver function or other side effects seen with ATRA or other retinoids (Muto et al., 1996). In this study, we show that NIK-333 promotes liver regeneration only at low doses. In contrast, ATRA dose-dependently stimulates liver regeneration (Fig. 2). Therefore, the mitogenic effect of ATRA must be cautioned regarding its possible use as an antitumoral drug (McCormick et al., 1990). Taken together, NIK-333 is a safe agent that selectively promotes proliferation of hepatocytes only at low doses, and suppresses liver oncogenesis at higher doses (Kagawa et al., 2004; Sano et al., 2005).

To evaluate the degree of liver injury after 70% partial hepatectomy in vivo, the activity of liver-related enzymes in serum such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed (Figs. 6 and 7). We show that serum ALT and AST activities increase rapidly and significantly after 70% partial hepatectomy on day 1, and return almost to pre-operative levels after 2 - 3 days in solvent-administered control rats. An explanation for this is that in response to damage around the ligated area of the liver, a local inflammatory reaction may occur, and transient increases in serum ALT and AST activities are thought to appear. Hepatic resection that induces an increase in serum ALT and AST activities was found to be significantly inhibited in animals treated with NIK-333, compared to the

solvent-administered controls, ATRA, or the retinol group (Figs. 6 and 7; Ledda-Columbano et al., 2004.). These results indicate that NIK-333 can significantly suppress the leakage of cytosolic enzymes from liver cells and prevent early stage liver damage caused by 70% partial hepatectomy. NIK-333 is likely to have an anti-inflammatory action similar to ibuprofen and dexamethasone (Kimura et al., 2008).

In conclusion, we show for the first time that chemically synthesized acyclic retinoid, NIK-333 in low concentrations is a potent stimulator of normal hepatocyte proliferation and restoration of liver function after 70% partial hepatectomy in rats in vivo. The underlying molecular mechanisms by which NIK-333 stimulates liver regeneration and restores liver function are still unknown at present. Hence, members of our laboratory are currently investigating such mechanisms in primary cultures of adult rat hepatocytes in vitro.

5. References

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Legends

Fig. 1. Time course-associated effects of NIK-333 and natural retinoids on liver regeneration after 70% partial hepatectomy.

Rats were treated according to the experimental schedule described in Materials and Methods. Control (soybean oil; 4.0 mL/kg, p.o.), NIK-333 (0.4 mg/kg, p.o.), ATRA (4.0 mg/kg, p.o.), or retinol (40 mg/kg, p.o.), were injected through an oral tube once daily (at 10 A.M.), after which the remnant livers were removed rapidly under ether anesthesia at the time points indicated, and weighed. In each experimental group n = 5-6 animals. The data is expressed as a percentage of the liver-to-body weight ratio on day 0. 100%: Sham-operated rats. Values are expressed as means±S.E.M. *, $P< 0.05$; **, $P<0.01$ compared to soybean oil-treated control.

Fig. 2. Dose-dependent effect of NIK-333 and natural retinoids on liver regeneration on day 3 after 70% partial hepatectomy.

Rats were treated according to the experimental schedule described in Materials and Methods. Control (soybean oil; 4.0 mL/kg, p.o.), NIK-333 (0.004-40 mg/kg, p.o.) with or without monoclonal antibody to TGF-β1 (10 mg/kg, i.p.), ATRA (0.004-40 mg/kg, p.o.), or retinol (0.004-40 mg/kg, p.o.), were injected through an oral tube once daily (at 10 A.M.), and the remnant livers were removed rapidly under ether anesthesia on day 3 after 70%

partial hepatectomy, and weighed. The ratio of total liver weight after regeneration normalized to body weight was used as an indicator of liver regeneration. The data is expressed as a percentage of the liver-to-body weight ratio on day 0. Rats (n=5-8) were used in each experimental group. Values are the means ± S.E.M. *, P< 0.05 ; **, P<0.01 compared to solvent-treated controls.

Fig. 3. Light micrographs showing typical liver sections from normal rats treated with or without administration of NIK-333 or ATRA on day 3 after 70% partial hepatectomy.

Histological changes in the livers of rats without any treatment (sham operation), or with a solvent (control), NIK-333, or ATRA, on day 3 after 70% partial hepatectomy were examined as described in Materials and Methods. Liver specimens were fixed in cold 10% neutralized formalin and embedded in paraffin. Paraffin-embedded tissue sections (5μm thick) were deparaffinized and stained with Hematoxylin and Eosin. A: sham operation, B: control (soybean oil; 4.0 mL/kg/day, p.o.), C: NIK-333, 0.4 mg/kg/day, p.o., D: ATRA, 4.0 mg/kg/day, p.o. Bar represents 50 μm.

Fig. 4. Distribution of hepatocytes undergoing DNA synthesis on day 1 after 70% partial hepatectomy.

Light micrographs showing typical distributions of hepatocytes undergoing DNA synthesis in remnant livers of rats treated without any treatment (sham operation) or with a solvent (control), NIK-333, or ATRA, on day 1 after 70% partial hepatectomy. The cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) in vivo. Liver sections (5μm thick) were stained with Hematoxylin following immunochemical staining with a monoclonal antibody to BrdU as described in Materials and Methods. Dark spots indicate hepatocytes undergoing DNA synthesis. A: Sham operation, B: Control (soybean oil, 4.0 mL/kg/day, p.o.), C: NIK-333, 0.4 mg/kg/day, p.o.), D: ATRA, 4.0 mg/kg/day, p.o. Bar represents 50 μm.

Fig.5. Time course-associated effects of NIK-333 and ATRA on 5-bromo-2'-deoxyuridine (BrdU) labeling indexes, on days 0 to 5 after 70% partial hepatectomy.

BrdU-mitotic indexes were determined by BrdU incorporation into DNA and from immunohistochemical staining using an anti-monoclonal antibody to BrdU on days 0-5 after partial hepatectomy, as described in the legend of Fig. 4. BrdU labeling index in solvent-, NIK-333-, and ATRA-treated rats indicates the percentage of BrdU-positive hepatocyte nuclei out of 1000 nuclei in a randomly selected field under a light microscope. Values are the means ± S.E.M. of five different rats. *, $P< 0.05$; **, $P<0.01$ compared to

solvent-treated control.

Fig. 6. Time course-associated effects of NIK-333 and natural retinoids on liver-specific cytosolic transaminase (ALT and AST) activities in serum after subjection to 70% partial hepatectomy.

Time course-associated effects of NIK-333 (0.4 mg/kg/day, p.o.), ATRA (4.0 mg/kg/day, p.o.), or retinol (40 mg/kg/day, p.o.), on serum ALT (A) and AST (B) activities assessed on days 0-14 after 70% partial hepatectomy, as described in Materials and Methods. Data from control, NIK-333-, ATRA-, and retinol-treated rats are shown as the mean ± S.E.M. (n=5~6). *, $P< 0.05$; **, $P<0.01$ compared to solvent-treated control.

Fig. 7. Dose-dependent effect of NIK-333 and natural retinoids on liver-related cytosolic transaminase (ALT and AST) activities in serum on day 3 after subjection to 70% partial hepatectomy.

Rats were treated according to the experimental schedule described in Materials and Methods. Serum ALT and AST activities were determined as described in the legend of Fig. 6. Each experimental group contained 5-6 rats. Values are represented as the means ± S.E.M. *, $P< 0.05$; **, $P<0.01$ compared to solvent-treated controls.

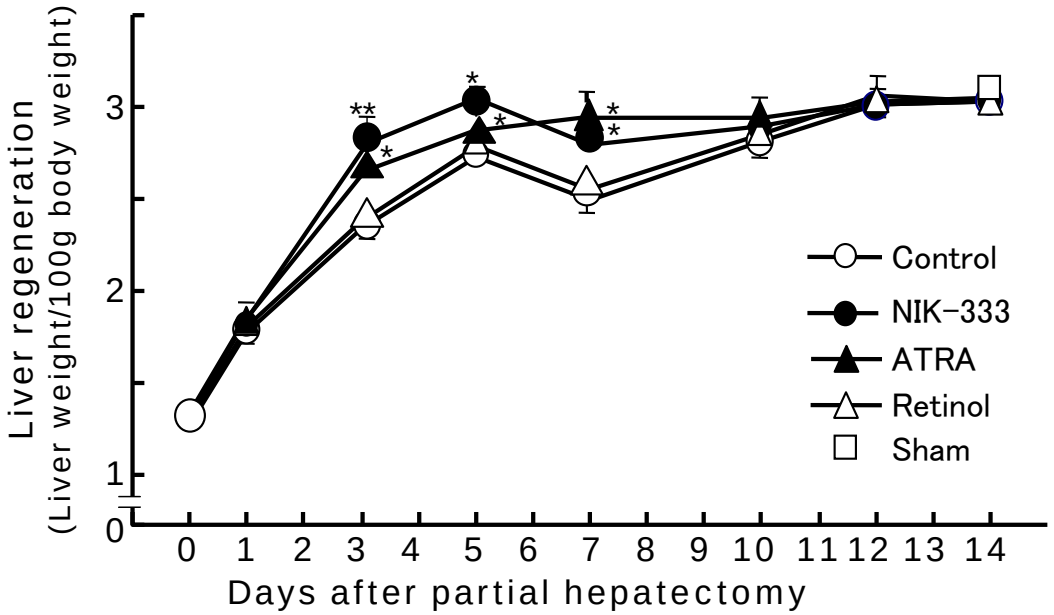

Fig.1
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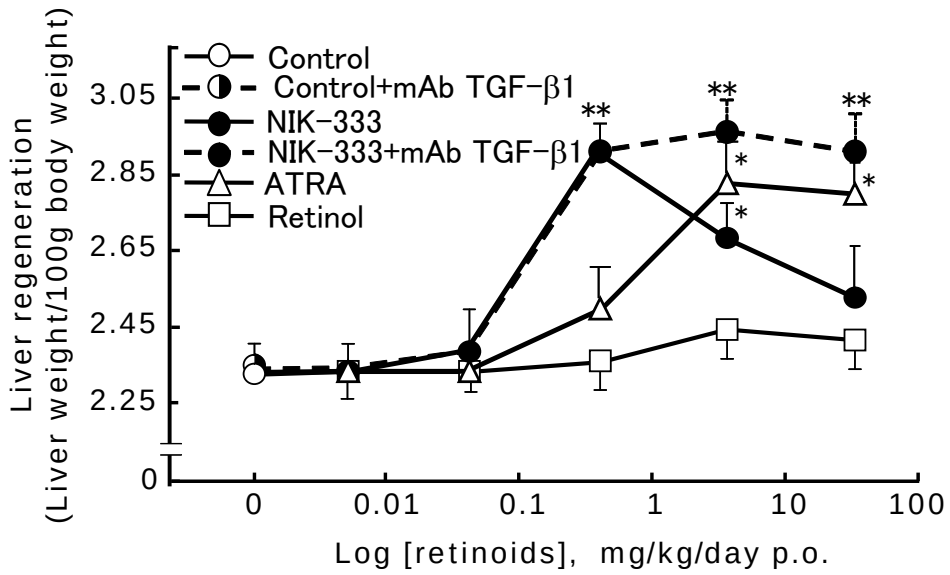

Fig.2
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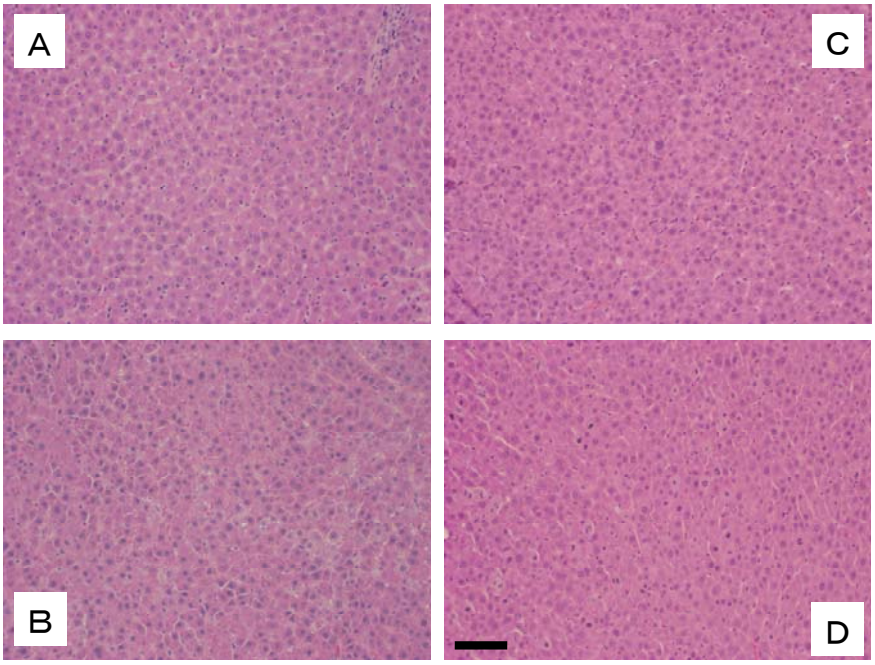

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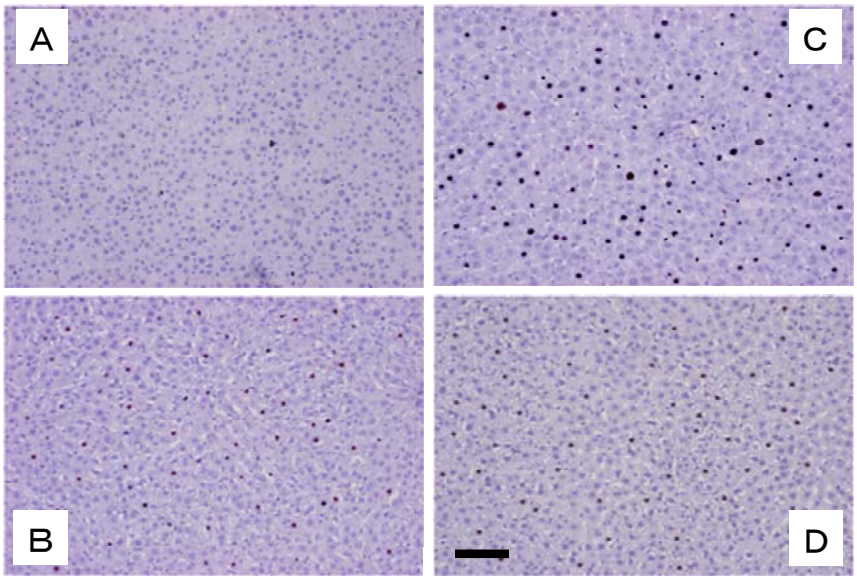

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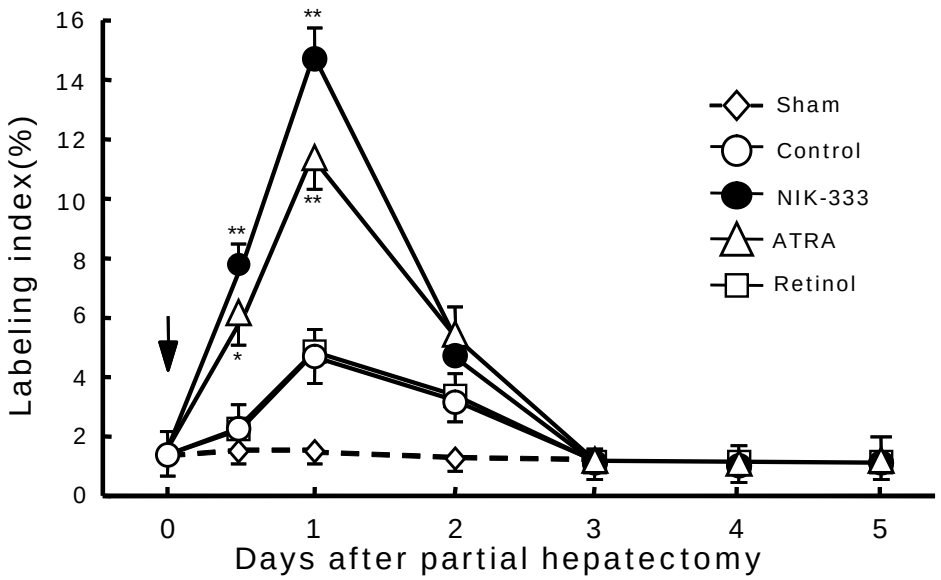

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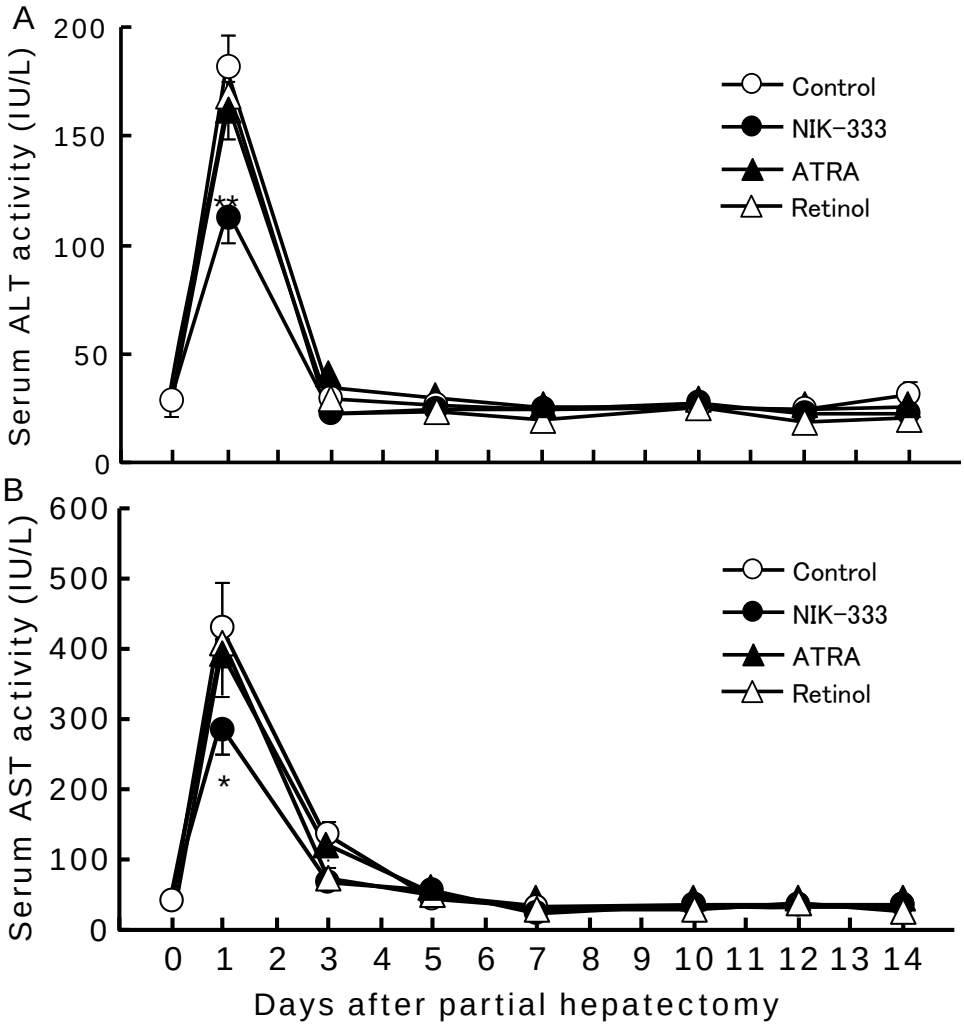

Fig.6
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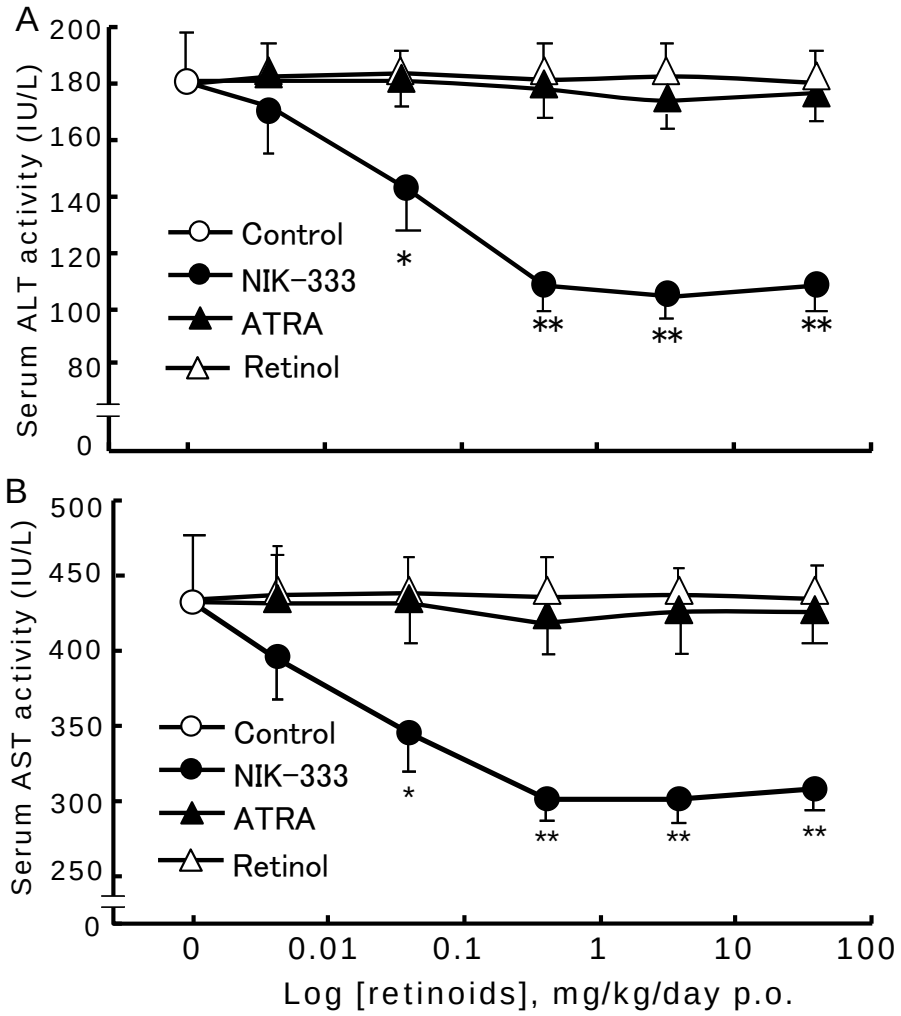

Fig.7
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